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(54) Title: NOVEL TRH RECEPTOR

(57) Abstract

The present invention is directed to the novel receptor for TRH which has been designated as THR receptor 2. The invention encompasses both the receptor protein as well as nucleic acids encoding the protein. In addition, the present invention is directed to methods and compositions which rely upon either TRHR-2 proteins or nucleic acids.

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NOVEL TRH RECEPTOR

Field of the Invention

The present invention is in the field of biological receptors and the various uses that can be made of such receptors. More specifically, it relates to nucleic acids encoding a novel receptor for thyrotropin-releasing hormone and to the receptor protein itself.

Background of the Invention

Thyrotropin-releasing hormone (TRH) is a tripeptide (pyroglutamic acid-histidine-proline-amide) present in the central nervous system (thalamus, cerebral cortex, spinal cord) as well as in the periphery (pancreas, gastrointestinal tract, placenta). In the hypothalamus, TRH is synthesized by peptidergic neurons of supraoptic and paraventricular nuclei. It is then axonally transported to the median eminence where it is stored. Upon secretion into the bloodstream, TRH is transported to the pituitary where it stimulates the production of thyroid stimulating hormone (TSH) which, in turn, stimulates the production of thyroxin (T4) in the thyroid gland (Gaillard, in Pharmacologie: Des Concepts Fondamentaux Aux Applications Thérapeutiques, M. Shorderet ed., pp. 415-448 (1992)).

In addition to its role in regulating the synthesis and secretion of hormones from the anterior pituitary, there is evidence that TRH acts as a neurotransmitter (Wu, *et al.*, *Neurosci. Let. 142*:143-146 (1992)). TRH is found abundantly in the central nervous system and exogenous administration of TRH elicits a variety of behavioral changes. It produces a rapid onset, neurotransmitter-like, excitation of spinal lower motor neurons and reduces neurological deficits observed after traumatic spinal cord injury in cats.

The distribution of TRH-containing cells, fibers or receptors suggests a potential role for TRH in the perception of noxious stimuli. Specifically, TRH is present in the periaqueducal gray (PAG), the nuclei raphe magnus (NMR), in the pallidus and dorsal horn of the spinal cord. TRH binding sites have been found in the brain, pituitary, dorsal and ventral horns of the spinal cord, and in peripheral tissues. When injected centrally (I.C.V. and I.C.), TRH induces a short lasting supraspinal antinociception. The analgesia induced by I.C.V. TRH injection is twice as great, on a molar basis, as that induced by morphine (Boschi, *et al.*, *Br. J. Pharmacol. 79*:85-92 (1983)). This antinociceptive effect is detected in models of chemically and mechanically, but not thermally, induced pain.

The actions of TRH are mediated by the stimulation of specific cell surface receptors. There is evidence that TRH receptors found in the pituitary transmit their signal to the cell interior through a G protein to trigger the inositol phospholipid-calcium-protein kinase C transduction pathway (Straub, *et al.*, *Proc. Nat'l. Acad. Sci. U.S.A. 87*:9514-9518 (1990); Duthie, *et al.*, *Mol. Cell. Endocrinol. 95*:R11-R15 (1993)). A cDNA sequence encoding a G protein-coupled TRH receptor was first isolated from mouse pituitary cells using an expression cloning strategy (Straub, *et al.*, *Proc. Nat'l. Acad. Sci. U.S.A. 87*:9514-9518 (1990)). Subsequently, several groups have described the cloning of rat TRH receptor cDNAs expressed in either a pituitary tumor cell line (GH3) or in pituitary gland (Duthie, *et al.*, *Mol. Cell. Endocrinol. 95*:R11-R15 (1993); De La Pena, *et al.*, *J. Biol. Chem. 267*:25703-25708 (1992)). In addition, two isoforms of the rat TRH receptor have been shown to be generated from a single gene by alternative splicing (De La Pena, *et al.*, *J. Biol. Chem. 267*:25703-25708 (1992)).

In addition to receptors isolated from the mouse and rat, a human TRH receptor cDNA has been cloned by Matre *et al.* (*Biochem. Biophys. Res. Comm. 195*:179-185 (1993)). With the exception of its C-terminal region, the predicted amino acid sequence of the human receptor was found to be more than 95% homologous to its counterparts from the rat and mouse.

Using synthetic TRH analogues, a dissociation of endocrine and CNS effects has been observed, suggesting that subtypes of TRH receptor may exist. Certain analogues were found to affect sleeping time and breathing frequency in test animals even though they failed to bind to pituitary or brain receptors and had no measurable TSH release activity (Alexandrova, *et al., Gen. Physiol. Biophys. 10*:287-297 (1991)). Other analogues, modified in the C-terminal region, have been identified which are ineffective in treating traumatic spinal cord injury but which maintain the same endocrine effects as normal TRH (Faden, *Brain Research 486*:228-235 (1989)).

The existence of distinct TRH receptor subtypes has also been suggested by biochemical experiments. Specifically, TRH receptors isolated from the brain were found to have an isoelectric point of 5.5 whereas those isolated from the pituitary were found to have an isoelectric point of only 4.9. One possible explanation for this difference is that the receptors in the brain and those in the pituitary have different amino acid sequences (Burt, *Ann. NY Acad. Sci. 553*:188 (1989)). In addition, electrophysiological experiments and measurements of intracellular calcium concentration have suggested that TRH and TRH metabolites present in the brain may act by binding to different subtypes of TRH binding sites (Toledo-Aral, *et al., J. Physiol. 472*:327-340 (1993)).

Therapeutically, it is clear that agonists and antagonists of TRH binding have potential value in regulating endocrine function, controlling pain, and in the treatment of spinal cord injury. The ability to identify such agents will depend upon the availability of purified TRH receptors suitable for binding assays. Such assays could be used to screen for TRH agonists and antagonists; to determine the extent to which a patient's plasma contains an appropriate level of binding activity; and to help monitor the purity and effectiveness of agents at all stages of drug development.

Summary of the Invention

To date, the only TRH receptor which has been cloned has been designated TRHR-1. The present invention is based upon the discovery of a new receptor for TRH which differs from TRHR-1 in terms of structure, tissue distribution and binding characteristics. Thus, in its first aspect, the invention is directed to a protein, except as existing in nature, comprising the amino acid sequence consisting functionally of the sequence of SEQ ID NO:2. The term "consisting functionally of" refers to proteins in which the sequence of SEQ ID NO:2 has undergone additions, deletions or substitutions which do not substantially alter the functional characteristics of the receptor. The term is intended to encompass proteins having exactly the same amino acid sequence as that of SEQ ID NO:2, as well as proteins with sequence differences that are not substantial as evidenced by their retaining the basic, qualitative ligand binding properties of TRHR-2.

The invention also encompasses substantially pure proteins with sequences consisting essentially of that of SEQ ID NO:2; antibodies that bind preferentially to such proteins (i.e., antibodies having at least a 100-fold greater affinity for TRHR-2 than any other protein); and antibodies made by a process involving the injection of a pharmaceutically acceptable preparation of TRHR-2 into an animal capable of antibody production. In a preferred embodiment, monoclonal antibody to TRHR-2 is produced by injecting the pharmaceutically acceptable preparation of TRHR-2 into a mouse and then fusing mouse spleen cells with myeloma cells.

The invention is also directed to a substantially pure polynucleotide encoding a protein comprising the amino acid sequence consisting functionally of SEQ ID NO:2. This aspect of the invention encompasses polynucleotides encoding proteins consisting essentially of the amino acid sequence of SEQ ID NO:2, expression vectors comprising such polynucleotides, and host cells transformed with such vectors. Also included is the recombinant TRHR-2 protein produced by host cells made in this manner. Preferably, the polynucleotide encoding TRHR-2 has the nucleotide sequence shown in SEQ ID NO:1,

and the vectors and host cells used for expression of TRHR-2 also use this particular polynucleotide.

In another aspect, the present invention is directed to a method for assaying a test compound for its ability to bind to TRHR-2. This assay is performed by incubating a source of TRHR-2 with a ligand known to bind to the receptor and with the test compound. The source of TRHR-2 should be substantially free of other types of TRH receptors such as TRHR-1, e.g., greater than 90% of the TRH receptors present in the source should correspond to TRHR-2. Upon completion of incubation, the ability of the test compound to bind to TRHR-2 is determined by the extent to which ligand binding has been displaced. The preferred ligand is either TRH or TRH which has been labelled with a detectable compound. The preferred source of TRHR-2 for use in the assay is a cell transformed with a vector for expressing the receptor and comprising a polynucleotide encoding a protein consisting essentially of the amino acid sequence of SEQ ID NO:2. Instead of using cells in the assay, a membrane preparation can be prepared from the cells and this can be used as a source of TRHR-2. Although not essential, the assay can be accompanied by a determination of changes in a second messenger, e.g. changes in the intracellular concentration of calcium. This should help to determine whether a test compound or analogue that binds to TRHR-2 is acting as an agonist or antagonist of TRH.

In another aspect, the present invention is directed to a method for assaying a test compound for its ability to alter the expression of the TRHR-2 gene. This method is performed by growing cells expressing TRHR-2, but substantially free of other TRH receptors, in the presence of the test compound. Cells are then collected and the expression of TRHR-2 is compared with expression of control cells grown under essentially identical conditions but in the absence of the test compound. In a preferred embodiment, the cells expressing TRHR-2 are cells transformed with an expression vector comprising a polynucleotide sequence encoding a protein consisting essentially of the amino acid sequence of SEQ ID NO:2. A preferred test compound is an oligonucleotide at least 15 nucleotides in length and comprising a sequence complimentary to a sequence shown in

SEQ ID NO:1. The preferred method for determining receptor expression is by means of a receptor binding assay.

Brief Description of the Drawings

Figure 1 (A and B): A: The nucleotide sequence and corresponding translated amino acid sequence (in single letter code) of rat TRHR-2 is shown. The nucleic acid sequence has been given the designation SEQ ID NO:1 and the amino acid sequence SEQ ID NO:2. Nucleotides are presented in the 5' to 3' orientation. B: The amino acid sequence of rat TRHR-2 is displayed starting with the initiating methionine.

Figure 2: The alignment of rat TRHR-2 with sequences of rat, mouse and human TRHR-1 are compared. Alignment was determined using the MacVector software from Kodak. The TRHR-2 sequence is shown in the top of the figure with the human (hTRHR-1), the mouse (mTRHR-1) and the rat (rTRHR-1) sequence below. An upper character indicates a perfect match whereas lower characters indicate mismatches. On the score line, (u) indicates a match with a score of +1 or greater, (-) indicates a score between -1 and +1, and (v) indicates a match of -1 or less, as determined using the pam250 scoring matrix of Dayhoff (see MacVector reference manual).

Figure 3: Figure 3 diagrammatically illustrates the cloning strategy that was used in obtaining and expressing TRHR-2.

Figure 4 (A and B): Figure 4A shows the TRHR-2 saturation curve. Saturation assays were performed as described in the text. Data from three distinct saturation experiments were analyzed using the non-linear least-squares regression curve-fitting program GraphPad Prism. TRH was found to bind with a K_d of 3.36 nM. The B_{max} for the assay was found to be 431.68 fmol/mg. Figure 4B shows the results of competition binding assays performed using various ligands and the TRHR-2 receptor. Data was again analyzed

using the GraphPad Prism program and a typical result is displayed in the figure. The mean IC_{50} for the various ligands is shown in Table 1.

Figure 5: Intracellular calcium concentration changes were determined by fluores-cence measurement of the intracellular, calcium-sensitive indicator Fura-2 as described in the text. TRH was incubated with wild type 293 cells (293(WT), Panel B) and with TRHR-2 transfected 293 cells (HEK293/TRHR-2, Panel A). The concentration of TRH is indicated in parentheses. As a control for the state of the 293(WT) cells, the response of the endogenous bradykinin receptor present in the cells was tested by incubating them in the presence of 100nM bradykinin (BK).

Figure 6: Figure 6 shows a Northern blot analysis of TRHR-2 mRNA expression in various tissues. A multiple-tissue Northern blot was performed with two µg of poly(A)$^+$ RNA from heart (lane 1), brain (lane 2), spleen (lane 3), lung (lane 4), liver (lane 5), skeletal muscle (lane 6), kidney (lane 7), and testes (lane 8). Hybridization was performed with a ^{32}P-labelled cDNA probe for TRHR-2. Only lane 2, containing RNA derived from the brain, showed a prominent band. This appeared to have a size of about 8 kb.

Figure 7: Figure 7 shows an *in situ* hybridization film comparing TRHR-1 and TRHR-2 mRNA expression in the central nervous system and pituitary gland of the rat. Consecutive sections from the sagittal brain (panel A), coronal brain (panel B), spinal cord at four different levels (panel C), and pituitary were hybridized with ^{35}S-labelled riboprobes to TRHR-1 and TRHR-2 mRNA. It can be seen that the pattern of TRHR-2 expression differs markedly from that of TRHR-1.

Definitions

The description that follows uses a number of terms that refer to recombinant DNA technology. In order to provide a clear and consistent understanding of the specification

and claims, including the scope to be given such terms, the following definitions are provided.

Cloning vector: A plasmid or phage DNA or other DNA sequence which is able to replicate autonomously in a host cell, and which is characterized by one or a small number of restriction endonuclease recognition sites. A foreign DNA fragment may be spliced into the vector at these sites in order to bring about the replication and cloning of the fragment. The vector may contain a marker suitable for use in the identification of transformed cells. For example, markers may provide tetracycline resistance or ampicillin resistance.

Expression vector: A vector similar to a cloning vector but which is capable of inducing the expression of the DNA that has been cloned into it, after transformation into a host. The cloned DNA is usually placed under the control of (i.e., operably linked to) certain regulatory sequences such as promoters or enhancers. Promoter sequences may be constitutive, inducible or repressible.

Substantially pure: As used herein, "substantially pure" means that the desired product is essentially free from contaminating cellular components. Contaminants may include, but are not limited to, proteins, carbohydrates or lipids. One method for determining the purity of a protein or nucleic acid is by electrophoresing a preparation in a matrix such as polyacrylamide or agarose. Purity is evidenced by the appearance of a single band after staining.

Host: Any prokaryotic or eukaryotic cell that is the recipient of a replicable expression vector or cloning vector is the "host" for that vector. The term encompasses prokaryotic or eukaryotic cells that have been engineered to incorporate a desired gene on its chromosome or in its genome. Examples of cells that can serve as hosts are well known in the art, as are techniques for cellular transformation (see e.g. Sambrook *et al.*, Molecular Cloning: A Laboratory Manual, 2nd ed. Cold Spring Harbor (1989)).

Promoter: A DNA sequence typically found in the 5' region of a gene, located proximal to the start codon. Transcription is initiated at the promoter. If the promoter is of the inducible type, then the rate of transcription increases in response to an inducing agent.

Complementary Nucleotide Sequence: A complementary nucleotide sequence, as used herein, refers to the sequence that would arise by normal base pairing. For example, the nucleotide sequence 5'-AGAC-3' would have the complementary sequence 5'-GTCT-3'.

Expression: Expression is the process by which a polypeptide is produced from DNA. The process involves the transcription of the gene into mRNA and the translation of this mRNA into a polypeptide.

Detailed Description of the Invention

The present invention is directed to the TRHR-2 receptor protein, genetic sequences coding for the receptor, a method for assaying compounds for their ability to bind to TRHR-2 and a method for assaying compounds for their ability to alter TRHR-2 expression. The receptor and the nucleic acids encoding the receptor may be distinguished from all known TRH receptors based upon structure, tissue distribution and binding characteristics. With respect to structure, the relationship between TRHR-2 and other TRH binders is shown in figure 2. The greatest homology was observed between TRHR-2 and the human TRHR-1 receptor. In transmembrane regions, the sequence homology between these receptors ranged from 52% to 80%. The alignment of TRHR-2 relative to other G protein-coupled receptors, or other members of the neuropeptide receptor subfamily, indicates a unique sequence indicative of a newly characterized receptor.

It will be understood that the present invention encompasses not only the sequence identical to that shown in figure 1, but also sequences that are essentially the same and which produce a receptor retaining the basic binding characteristics of TRHR-2. Thus, the invention relates to proteins comprising amino acid sequences consisting functionally of

the sequence of SEQ ID NO:2. In this regard, it is well known that techniques such as site-directed mutagenesis may be used to introduce variations in a protein structure. Variations in TRHR-2 introduced by this or some similar method are encompassed by the invention to the extent that such variant receptors retain the ability to preferentially bind to TRH or TRH-like peptides.

The TRHR-2 receptor may also be distinguished from similar proteins based upon its binding characteristics. pGlu-His-Pro-Gly does not show any binding to GH4C1 cells but displays a Ki value of 640 nM to the TRHR-2 receptor. This suggests that TRHR-2 is less susceptible than TRHR-1 to C-terminal modification of the ligand.

In addition, TRHR-2 may be distinguished from other receptors for TRH based upon its tissue distribution. *In situ* hybridization studies performed on the rat have indicated a distinct distribution of TRHR-2 in the pituitary gland, spinal cord, and brain. In the pituitary gland, moderate levels of TRHR-1 mRNA have been observed throughout the anterior lobe whereas both the posterior and intermediate lobes appear to be devoid of expression. In contrast, no specific hybridization signal was detected for TRHR-2 in the pituitary.

In the CNS, TRHR-2 mRNA is distributed throughout the entire dorsal horn of the spinal cord whereas TRHR-1 is located in sparsely distributed neurons of the ventral horn (Zabavnik, *et al.*, *Neuroscience 53*:877-887 (1993)). This is consistent with experiments in which autoradiography was used to detect ^{3}H-TRH binding sites and which suggest that TRH receptors are expressed in both the dorsal and ventral horns of the spinal cord (Manaker, *et al.*, *J. Neurosci. 5*:167-174 (1985)). In the brain, it appears that TRHR-2 mRNA is present in much higher levels than the mRNA for TRHR-1. In particular, TRHR-1 mRNA expression was observed only at very low levels in the piriform cortex, amygdala and discreet hypothalamic nuclei (superchiasmatic nucleus, SCN; ventromedial hypothalamic nucleus, VMH; paraventricular hypothalamic nucleus, PVN; and anterior hypothalamic area posterior part, AHP). In no case, with the possible exception of the

amygdala, was TRHR-1 mRNA detected in regions enriched in TRHR-2 such as the thalamus, medial habenular nucleus, frontal and parietal cortices, the pontine nucleus, or the cerebellum.

The pattern of TRHR-2 expression within the rat CNS suggests the involvement of at least two distinct modalities: somatosensory (possibly including pain transmission) and motor. The restricted localization of TRHR-2 mRNA throughout the entire dorsal horn of the spinal cord, reticular formation and somatosensory nuclei of the thalamus (VPL, VPM) is consistent with ascending pathways such as the spinothalamic and trigeminothalamic tracts (pain and crude touch) as well as the medial lemniscal system (discriminative touch). The presence of high levels of TRHR-2 restricted to the pontine nucleus and the cerebellum is consistent with a role in motor control and/or proprioception. These receptors may also be the anatomical substrate for the previously described TRH effects on motor control (see Engle, *et al.*, *The Lancet 83841*:73-75 (1983)). To date, only very low levels of TRH peptide or TRH binding sites have been reported in the cerebellum suggesting that an alternate ligand, as yet unidentified, may also bind to this receptor.

I. Nucleic Acids Coding for TRHR-2

As discussed above, DNA sequences coding for TRHR-2 are expressed in a variety of tissues, any of which may serve as a source for the isolation of nucleic acid coding for the receptor. The preferred source is the spinal cord of the rat, but spinal cord tissue from other species may be used as well. In addition, cells or cell lines expressing TRHR-2 may serve as a source for nucleic acid. These may either be cultured cells that have not undergone transformation or cell lines specifically engineered to express recombinant TRHR-2.

Many methods are available for isolating DNA sequences and may be adapted for the isolation of TRHR-2 nucleic acid (see for example Sambrook, *et al.*, Molecular Cloning: A Laboratory Manual, 2nd ed., Cold Spring Harbor Press (1989)). As discussed in the

Examples, a preferred method of isolation is to use reverse transcription PCR on mRNA isolated from rat spinal cord to produce probes and to then use these probes to screen a cDNA library. The preferred primers for carrying out the PCR amplification are:

TM3-4: 5'-AT(C or T)(A or G)(C or G)(C or T)(A or G)TIGAI(A or C)G(A or G)TA-3' (SEQ ID NO:3)

TM7-4: 5'-(A or C)(A or T)GG(C or T)(A or G)TAGAI(C or G)AI(A or C)GG(A or G)TT-3' (SEQ ID NO:4)

After having produced cDNA by reverse transcription, the above primers are used for amplification. This should result in partial amplification of the THRH2 cDNA and produce fragments suitable for screening cDNA libraries.

Although the procedure above is known to be suitable for obtaining TRHR-2 nucleic acid, it is expected that alternative techniques can be developed with relatively little effort. Thus, cDNA libraries may be screened using probes synthesized based upon the TRHR-2 sequence shown in figure 1. In general, probes should be at least 14 nucleotides long and should not be selected from regions known to be highly conserved among proteins, e.g., the transmembrane domains of G-protein linked receptors. Alternatively, using the sequence shown in figure 1, it should be possible to select different PCR primers and amplify the full length TRHR-2 sequence. The same techniques that have proven successful in the rat can be used to obtain TRHR-2 sequences from other species, e.g., from cells or tissues derived from humans.

II. Production and Isolation of TRHR-2 Recombinant Protein

In order to express recombinant TRHR-2, a DNA encoding the structural sequence for the protein described above must be placed in a vector containing transcriptional and translational signals recognizable by an appropriate host. The cloned TRHR-2 sequences,

preferably in double-stranded form, are inserted into the expression vector in operable linkage, i.e., they are positioned so as to be under the control of the vector's regulatory sequences and in such a manner that mRNA is produced that is translated into the TRHR-2 amino acid sequence.

Expression of the TRHR-2 receptor protein in different hosts may result in different post-translational modifications that can, potentially, alter the properties of the receptor. Preferably, nucleic acid encoding TRHR-2 is expressed in eukaryotic cells, especially mammalian cells. These cells provide post-translational modifications which, *inter alia*, aid in the correct folding of the receptor protein. An appropriate vector, pCDNA3-THR2, and host, HEK293 cells, are described under "Examples."

Other mammalian cells that may be used include, without limitation, NIH-3T3 cells, CHO cells, HeLa cells, LM(tk$^-$) cells, etc. Vectors suitable for use in each of the various cell types are well known in the art (see e.g., Sambrook, *et al.*, *supra*). Preferred eukaryotic promoters include that of the mouse metallothionein I gene; the TK promoter of Herpes virus; the SV40 early promoter; and the yeast GAL4 gene promoter. Some examples of suitable prokaryotic promoters include those capable of recognizing T4 polymerases, the P_R and P_L promoters of bacteriophage lambda, and the trp, recA, heat shock and lacZ promoters of *E coli*. Expression vectors may be introduced into host cells by methods such as calcium phosphate precipitation, microinjection or electroporation. Cells expressing the TRHR-2 receptor can be selected using methods well known in the art. One simple method for confirming the presence of the receptor nucleic acid in cells is to perform PCR amplification using the procedures and primers discussed above. The presence of functional receptor may be confirmed by performing binding assays using labelled TRH.

Once cells producing recombinant TRHR-2 receptor have been identified, they may be used in either binding assays or in assays designed to identify agents capable of altering TRHR-2 expression. Alternatively, membranes may be isolated from the cells and these may be used in receptor binding assays.

III. Antibodies to TRHR-2

The present invention is also directed to antibodies that bind preferentially to TRHR-2 and to a process for producing such antibodies. Antibodies that "bind specifically to TRHR-2" are defined as those that have at least a 100-fold greater affinity for TRHR-2 than for any other protein, including TRHR-1. The process for producing such antibodies may involve either injecting the TRHR-2 protein itself into an appropriate animal or, preferably, injecting short peptides made to correspond to different regions of TRHR-2. The peptides should be at least five amino acids in length and should be selected from regions believed to be unique to the TRHR-2 protein. Thus, highly conserved transmembrane regions should generally be avoided in selecting peptides for the generation of antibodies.

Methods for making and detecting antibodies are well known to those of skill in the art as evidenced by standard reference works such as: Harlow, *et al.*, Antibodies: A Laboratory Manual, Cold Spring Harbor Laboratory, N.Y. (1988); Klein, Immunology: The Science of Self-Nonself Discrimination (1982); Kennett, *et al.*, Monoclonal Antibodies and Hybridomas: A New Dimension in Biological Analyses (1980); and Campbell, "Monoclonal Antibody Technology," in Laboratory Techniques in Biochemistry and Molecular Biology (1984)).

"Antibody," as used herein, is meant to include intact molecules as well as fragments which retain their ability to bind to antigens (e.g., Fab and $F(ab')_2$ fragments). These fragments are typically produced by proteolytically cleaving intact antibodies using enzymes such as papain (to produce Fab fragments) or pepsin (to produce $F(ab')_2$ fragments). The term "antibody" also refers to both monoclonal and polyclonal antibodies. Polyclonal antibodies are derived from the sera of animals immunized with the antigen. Monoclonal antibodies can be prepared using hybridoma technology (Kohler, *et al.*, *Nature* 256:495 (1975); Hammerling, *et al.* in Monoclonal Antibodies and T-Cell Hybridomas, Elsevier, N.Y., pp. 563-681 (1981)). In general, this technology involves immunizing an

animal, usually a mouse, with either intact TRHR-2 or a fragment derived from TRHR-2. The splenocytes of the immunized animals are extracted and fused with suitable myeloma cells, e.g., SP_2O cells. After fusion, the resulting hybridoma cells are selectively maintained in HAT medium and then cloned by limiting dilution (Wands, *et al.*, *Gastroenterology 80*:225-232 (1981)). The cells obtained through such selection are then assayed to identify clones which secrete antibodies capable of binding TRHR-2.

The antibodies, or fragments of antibodies, of the present invention may be used to detect the presence of TRHR-2 protein using any of a variety of immunoassays. For example, the antibodies may be used in radioimmunoassays or immunometric assays, also known as "two-site" or "sandwich" assays (see Chard, T., "An Introduction to Radioimmune Assay and Related Techniques," in Laboratory Techniques in Biochemistry and Molecular Biology, North Holland Publishing Company, N.Y. (1978)). In a typical immunometric assay, a quantity of unlabelled antibody is bound to a solid support that is insoluble in the fluid being tested, e.g., blood, lymph, cellular extracts, etc. After the initial binding of antigen to immobilized antibody, a quantity of detectably labelled second antibody (which may or may not be the same as the first) is added to permit detection and/or quantitation of bound antigen (see e.g. Radioimmune Assay Method, Kirkham, *et al.*, ed. pp. 199-206 (E&S. Livingstone, Edinburgh (1970)). Many variations of these types of assays are known in the art and may be employed for the detection of TRHR-2.

Antibodies to TRHR-2 may also be used in the purification of either the intact receptor or fragments of the receptor (see generally, Dean, *et al.*, Affinity Chromatography, A Practical Approach, IRL Press (1986)). Typically, antibody is immobilized on a chromatographic matrix such as Sepharose 4B. The matrix is then placed in a column and the preparation containing TRHR-2 is passed through under conditions that promote binding, e.g., under conditions of low salt. The column is then washed and bound TRHR-2 is eluted using a buffer that promotes dissociation from antibody, e.g., buffer having an altered pH or salt concentration. The eluted TRHR-2 may then be transferred into a buffer of choice, e.g., by dialysis, and either stored or used directly.

IV. Assay for TRHR-2 Binding

One of the main uses for TRHR-2 nucleic acids and recombinant proteins is in assays designed to identify agents, other than TRH, capable of binding to the TRHR-2 receptor. Such agents may either be agonists, mimicking the effects of TRH, or antagonists, inhibiting the effects of TRH. Of particular interest is the identification of agents which bind to TRHR-2 receptors and increase the intracellular concentration of calcium in the cells. These agents have potential therapeutic application as analgesics; anesthetics; for reducing the damage due to spinal trauma; for controlling endocrine function; and for regulating gastric secretion, particularly in the treatment of ulcers.

An example of an assay that may be used for detecting compounds binding to TRHR-2 is presented in the examples and typical binding curves that may be obtained are shown in figure 4. The essential feature of the assays is that a source of TRHR-2 is incubated together with a ligand known to bind to the receptor and with a compound being tested for binding activity. The preferred source for TRHR-2 is cells, preferably mammalian cells, transformed recombinantly to express the receptor. The cells selected should not express a substantial amount of any other receptor which binds TRH, e.g., TRHR-1. This can easily be determined by performing TRH binding assays on cells derived from the same tissue or cell lines as those recombinantly expressing TRHR-2 but which have not undergone transformation.

The assay may be performed either with intact cells or, alternatively, with membranes prepared from the cells (see e.g., Wang, *et al.*, *Proc. Natl. Acad. Sci. USA 90*:10230-10234 (1993)). The membranes are incubated with a ligand specific for TRHR-2 and with a preparation of the compound being tested. After binding is complete, receptor is separated from the solution containing ligand and test compound, e.g., by filtration, and the amount of binding that has occurred is determined. Preferably, the ligand used is TRH detectably labelled with a radioisotope. However, fluorescent or chemiluminescent labels can be used

instead. Among the most commonly used fluorescent labelling compounds are fluorescein, isothiocynate, rhodamine, phycoerythrin, phycocycanin, allophycocyanin, *o*-phthaldehyde and fluorescamine. Useful chemiluminescent compounds include luminol, isoluminol, theromatic acridinium ester, imidazole, acridinium salt, and oxalate ester. Any of these agents which can be used to detectably label TRH will produce a ligand suitable for use in the assay.

Nonspecific binding may be determined by carrying out the binding reaction in the presence of a large excess of unlabelled ligand. For example, labelled TRH may be incubated with receptor and test compound in the presence of a thousandfold excess of unlabelled TRH. Nonspecific binding should be subtracted from total binding, i.e., binding in the absence of unlabelled TRH, to arrive at the specific binding for each sample tested. Other steps such as washing, stirring, shaking, filtering and the like may be included in the assays as necessary. Typically, wash steps are included after the separation of membrane-bound ligand from ligand remaining in solution and prior to quantitation of the amount of ligand bound, e.g., by counting radioactive isotope. The specific binding obtained in the presence of tests compound is compared with that obtained in the presence of labelled ligand alone to determine the extent to which the test compound has displaced TRH.

In performing binding assays, care must be taken to avoid artifacts which may make it appear that a test compound is interacting with the TRHR-2 receptor when, in fact, binding is being inhibited by some other mechanism. For example, the compound being tested should be in a buffer which does not itself substantially inhibit the binding of TRH to TRHR-2 and should preferably be tested at several different concentrations. Preparations of test compound should also be examined for proteolytic activity, and it is desirable that antiproteases be included in assays. Finally, it is desirable that compounds identified as displacing the binding of ligand to TRHR-2 receptor be re-examined in a concentration range sufficient to perform a Scathard analysis on the results. This type of analysis is well known in the art and can be used for determining the affinity of a test compound for receptor (see e.g., Ausubel, *et al.*, Current Protocols in Molecular Biology, 11.2.1-11.2.19

(1993); Laboratory Techniques in Biochemistry and Molecular Biology, Work, *et al.*, ed., N.Y. (1978), etc.). Computer programs may be used to help in the analysis of results (see e.g., Manson, *Methods Enzymol. 92*:543-577 (1983); McPherson, Kinetic, EBDA Ligand, Lowry-A Collection of Radioligand Binding Analysis Programs, Elsevier-Biosoft, U.K. (1985)). An example of the type of curve that may be obtained using this method is shown in figure 4, and examples of inhibitory constants for TRH-related peptides determined using binding assays are given in the text.

Assays for determining changes in second messenger, e.g. changes in intracellular calcium concentration, may be performed using compounds that have been identified as a result of their ability to bind to TRHR-2. These assays may be carried out as discussed in the examples or using other methods for determining intracellular calcium concentration. Typically, calcium concentration assays will be performed separately from binding assays, but it may also be possible to perform binding and calcium concentration assays on a single preparation of cells. TRHR-2 binding compounds that stimulate an increase in intracellular calcium in cells are agonists of TRH and should mimic its biological effects. In contrast, compounds that specifically bind to TRHR-2 receptors but which do not increase intracellular calcium are antagonists of TRH and should inhibit its biological effects.

V. Assay for Ability to Modulate TRHR-2 Expression

One way to either increase or decrease the biological effects of TRH is to alter the extent to which TRHR-2 is expressed in cells. Therefore, assays for the identification of compounds that either inhibit or enhance expression of TRHR-2 are of considerable interest. These assays are carried out by growing cells expressing TRHR-2 in the presence of a test compound and then comparing receptor expression in these cells with cells grown under essentially identical condition but in the absence of the test compound. As in the binding assays discussed above, it is desirable that the cells used be substantially free of receptors for TRH other than TRHR-2. Scatchard analysis of binding assays performed with detectably labelled TRH can be used to determine receptor number.

The binding assays may be carried out as discussed above in section IV and will preferably utilize cells that have been engineered to recombinantly express TRHR-2 as described in sections I and II. Ideally, the expression of TRHR-2 protein is controlled by the naturally occurring TRHR-2 regulatory element, e.g., the promoter which regulates cellular TRHR-2 expression *in vivo*.

A preferred group of test compounds for inclusion in the TRHR-2 expression assay consists of oligonucleotides complimentary to various segments of the TRHR-2 nucleic acid sequence. These oligonucleotides should be at least 15 bases in length and should be derived from non-conserved regions of the receptor nucleic acid sequence.

Oligonucleotides which are found to reduce receptor expression may be derivatized or conjugated in order to increase their effectiveness. For example, nucleoside phosphorothioates may be substituted for their natural counterparts (see Cohen, Oligodeoxynucleotides, Antisense Inhibitors of Gene Expression, CRC Press (1989)). The oligonucleotides may be delivered to a patient *in vivo* for the purpose of inhibiting TRHR-2 expression. When this is done, it is preferred that the oligonucleotide be administered in a form that enhances its uptake by cells. For example, the oligonucleotide may be delivered by means of liposome or conjugated to a peptide that is ingested by cells (see e.g., U.S. Patent No. 4,897,355 and 4,394,448; see also non-U.S. patent documents WO 89/03849 and EP0263740). Other methods for enhancing the efficiency of oligonucleotide delivery are well known in the art and are also compatible with the present invention.

Having now described the invention, the same will be more readily understood through reference to the following examples which are provided by way of illustration and which are not intended to limit the scope of the invention.

Examples

Example 1: Cloning and Sequencing of a Rat (TRHR-2) Thyrotropin-Releasing Hormone Receptor

A. Cloning and Sequencing Procedures

In order to isolate novel cDNA sequences encoding G protein-coupled receptors, a PCR-based homology screening strategy was used. Rat spinal cord mRNA was isolated using the FastTrack™ kit (InVitrogen, San Diego, Ca). Candidate sequences likely to encode G protein-coupled receptors were amplified from this mRNA by reverse transcription PCR using the following primers:

TM3-4: 5'-AT(C or T)(A or G)(C or G)(C or T)(A or G)TIGAI(A or C)G(A or G)TA-3' (SEQ ID NO:3)

TM7-4: 5'-(A or C)(A or T)GG(C or T)(A or G)TAGAI(C or G)AI(A or C)GG(A or G)TT-3' (SEQ ID NO:4)

The templates for PCR amplification were synthesized using GeneAmp RNA PCR kits (N808-0017 Perkin Elmer) together with 200 ng of spinal cord poly A^+ RNA. One aliquot of the produced cDNA was then amplified with 200 pmoles each of TM3-4 and TM7-4 primers and 2.5 units of Taq DNA polymerase in 50 mM KCl, 1.5 mM $MgCl_2$, 10 mM Tris(HCl), 200 μM dNTPs, pH 9.0. The reaction tubes were heated at 95 degrees C for one minute and subjected to 39 cycles of denaturation (95 degrees C/min), annealing (42 degrees C/min) and extension (72 degrees C/min).

The amplified fragments were analyzed and size fractionated on a 1% agarose gel. Fragments between 500 bp and 800 bp were excised from the gel, purified using the Sephaglas BandPrep™ kit from Pharmacia (cat# 27-9285-01), and inserted into the

pGEM-T vector from Promega (cat# A3600). Recombinant pGEM-T clones were selected randomly and plasmid DNA was prepared using the alkaline lysis method starting with 10 ml of bacterial culture. The DNA sequence from these clones was determined using the Sanger dideoxynucleotide chain termination method on denatured double-stranded plasmid templates (Sanger *et al.*, *Proc. Natl. Acad. Sci. USA 74*:5463-5437 (1977)), using the T7 sequencing kit from Pharmacia (cat# 27-1682-01). The clone pGemT-1-75 showed marked sequence homologies with known GPCRs. The most homologous sequence was the human and rat thyrotropin release hormone receptors (TRHR) (Matre *et al.*, *Biochem. Biophys. Res. Comm. 195*:179-185 (1993); Hinuma *et al.*, *Bioch. Biophys. Acta 1219*:251-259 (1994); Duthie *et al.*, *Mol Cell. Endocrinol. 95*:R11-R15 (1993)), although a perfect match was not identified.

The insert DNA fragment of clone pGemT-1-75 was excised from the vector using Pst I and Sac II, isolated from an agarose gel and labeled with ^{32}P by random primed synthesis using the Ready-To-Go™ DNA labeling kit (27-9251-01) from Pharmacia (Sambrook *et al.*, Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory (2nd ed. 1989)). This probe was used to screen a rat brain stem-spinal cord cDNA library in λ ZAP II (Stratagene, cat# 936521). The filters were incubated with the probe for 18 hours at 65°C in 2x SSC, 5x Denhardt's solutions and 0.2% SDS. The filters were rinsed twice in 0.1x SSC, 0.2% SDS at room temperature. They were then washed twice for 45 min in 0.1x SSC, 0.2% SDS at 65°C, once for 45 min at 65°C in 5 mM EDTA, 0.2% SDS, pH 8.0 and finally using 0.1x SSC at room temperature.

Hybridization-positive phages were purified and their inserts rescued by helper phage mediated excision to yield plasmid DNA (Murray *et al.*, *Mol. Gen. Genet. 150*:53-61 (1977); Schweinfest *et al.*, *Genet. Anal. Tech. Appl. 7*:64-70 (1990)). The insert of plasmid pBS/TRHR2 was sequenced after having generated a series of overlapping clones using the Erase-A-Base kit from Promega (cat# 65740).

B. Results

An open reading frame of 352 amino acids was detected flanked by 3' and 5' untranslated regions of, respectively, 183 and 361 bp. The sequence of the open reading frame is displayed in figure 1. The relative molecular mass of the predicted protein is 39,500 daltons. Hydropathy analysis of the encoded protein is consistent with a topography of seven transmembrane domains indicative of the G protein-coupled receptor family (Sprengel *et al.*, "Hormone Receptors," in Handbook of Receptors and Channels: G Protein-Coupled Receptors, pp. 153-207, CRC Press (1994)). In addition, sequence analysis revealed that the open reading frame of clone pBS-TRHR2 contains several conserved structural features found among the members of the neuropeptide receptor family, including: an asparagine in TM1 (Asn40); a leucine (Leu64) and an aspartic acid (Asp 68) in TM2; and a serine (Ser109), an arginine (Arg120) and a tyrosine residue (Tyr121) in TM3. Other features of this TRHR-2 receptor gene are the presence of a potential site for N-glycosylation in the amino terminus (Asn6) and the presence of several serines and threonines in the carboxyl terminus and third intracellular loop, which may serve as potential sites for phosphorylation by protein kinases.

A sequence comparison of the TRHR-2 open reading frame with the sequences of known TRH receptors is shown in Figure 2. The overall sequence homology between TRHR-2 and the known rat TRH receptor is 50.6%. However the sequence homology is higher in the putative transmembrane domains. Respectively, the homologies between the known rat TRH receptor (TRHR-1) and TRHR-2 in TM1 to TM7 are 61%, 80%, 74%, 58%, 52%, 77% and 71%.

Example 2: Transient Transfection Experiments

To generate a mammalian expression vector, a 1.3 Kb StuI - XbaI restriction fragment, from pBS/TRHR2 was isolated and subcloned between the Xba I and Eco RV sites of pCDNA3 (InVitrogen, San Diego, Ca). This expression vector was called pCDNA3-

TRHR2. Plasmid DNA for further analysis was prepared using the Qiaprep system from Qiagen.

HEK293s cells were obtained form the Cold Spring Harbor laboratory. They were inoculated in 6-well plates ($4x10^5$ cells per well) or in 10 cm Petri dishes (2×10^6 cells per dish) in Dulbeco's Modified Essential Medium (DMEM, Gibco BRL, cat# 11995-032) supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin, 100 μg/ml streptomycin and 0.25 μg/ml fungizone. One day after inoculation, the cells were transiently transfected using a modified $CaCl_2$ method. Three and a half mg of plasmid DNA per well or 20 μg per 10 cm petri dish was used. The cells were harvested 48 hours post transfection for ligand binding or signal transduction experiments. When transfected into HEK293 cells, pCDNA3-TRHR2 generated the expression of specific ^{3}H-TRH binding sites. No specific ^{3}H-TRH binding sites were generated by the transfection of the vector itself or a control pCDNA3 expression construct encoding an opioid receptor.

Example 3: Radioligand Binding To Stably Transfected Cells

A. Binding Assays

The TRH binding assay was performed on whole cells. Transfected cells were washed twice with HBSS (Gibco BRL, cat# 14065) supplemented with 0.05% bovine serum albumin, detached by gentle pipetting and aliquoted in eppendorf tubes for binding assays. One twentieth of the cells collected from a confluent 75 cm^2 flask was used per assay point. The binding reaction was performed in a total volume of 300 ml of binding buffer (HBSS + 0.05% bovine serum albumin) containing the transfected cells and 10 nM of ^{3}H-thyrotropin releasing hormone (Peninsula Laboratories Inc, cat # 7501) with or without unlabelled competitors. Non-specific binding was estimated in the presence of 1 mM of unlabelled TRH. Reactions were carried out for 60 min at room temperature and reactions were stopped by filtration through Unifilters-96 GF/B filters (Canberra Packard cat # 6005177) using the 96-well Filtermat 196 filtration system from Canberra Packard. The

filters were washed 3 times with 1 ml of washing buffer (50 mM Tris(HCl), 3 mM $MgCl_2$, pH 7.0). They were then dried at 55 °C for one hour and 50 µl of mScint-20 (Canberra Packard) was added per well. Filters were counted with the Topcount microplate counter from Canberra Packard.

B. Production of Stably Transfected Cells

HEK293s cells in a 10 cm petri dish were transfected with 20 mg pCDNA3-TRHR2. After 14 days of selection in culture medium containing 600 µg/ml G418, resistant colonies were pooled. Then, single clones were purified by 2 rounds of limited dilution in 96 well plates. Clones of HEK293s cells expressing different levels of TRHR-2 receptor were selected using a 3H-TRH binding assay.

C. Results

An example of a binding reaction performed using HEK293 cells expressing TRHR-2 is shown in figure 4. A single class of saturable ^{3}H-TRH binding site was detected displaying an estimated Kd for ^{3}H-TRH of 2.5 nM and a B_{max} of 430 fmol/mg proteins. Various TRH-related peptides were used in competition experiments. These experiments were performed using ^{3}H-TRH as a tracer and revealed IC_{50} values of 2.2 nM for pGlu-3 methyl-His-Pro-amide, 8.3 nM for TRH, 640 nM for pGlu-His-Pro-Gly, and >10000 nM for pGlu-Glu-Pro-amide (figure 4B and Table 1).

Table 1: Binding Parameters of TRH Receptors*

	293S/TRH-R clonal cell line	GH4C1 Cells
B_{MAX} (fmol/mg)	534.56 ± 70.82	577.48 ± 80.77
Kd (nM): ^{3}H-TRH	6.17 ± 1.51	5.32 ± 1.63
IC_{50} (nM): TRH (pGlu-His-Pro-amide)	8.39 ± 2.31	5.33 ± 2.81
IC_{50} (nM): pGlu-Glu-Pro-amide	»10000	»10000
IC_{50} (nM): pGlu-His-Pro-Gly	640.67 ± 75.19	»10000
IC_{50} (nM): pGlu-3-methyl-His-Pro-amide	2.20 ± 0.71	0.43 ± 0.27

* Three independent experiments were performed, each with duplicate data points.

Example 4: Functional Assay: Intracellular Calcium Mobilization

A. Assay Procedure

Intracellular calcium concentration changes where determined by fluorescence measurement of the intracellular, calcium-sensitive indicator Fura 2. Briefly, HEK293s cells were grown on glass cover slips in culture medium at 37°C, 5% CO_2 and diluted 10 fold every 3 days. The cells were loaded at room temperature for 30 min using 2 mM of Fura 2/AM in simplified Grace's solution (SGS buffer) physiological buffer (NBS: 135 mM NaCl, 5 mM KCl, 1.8 mM $CaCl_2$, 1.2 mM $MgSO_4$, 10 mM HEPES, pH 7.4). Fura 2/AM is a membrane permeant, calcium-insensitive ester of Fura 2. After three washes in Fura-2 /AM-free buffer, the cells were incubated for 15 to 30 min in SGNBS at room temperature to insure full hydrolysis of the Fura 2 ester. The calcium-sensitive, hydrolyzed

form of Fura 2 remains trapped intracellularly. Experiments were conducted at room temperature on single cells or small groups of 3 to 7 cells in a coverslip holder fitted to the stage of an IMT-2 inverted microscope equipped with a 40x-epifluorescence objective (UVFL40, 0.85, Olympus Optical Co, Tokyo) and coupled to a PTI ratio fluorescence system (Photon Technology International, London, Ontario, Canada). Sample illumination was provided by a 75W xenon light source attached to a filter-based light chopper unit which provided 340 and 380 nm excitation wavelength alternating at a frequency of 5 Hz. The emitted light was passed through an adjustable rectangular aperture followed by a 505 nm interference filter (10-nm bandwidth), and its intensity was recorded by a photon counter detector. Dye leakage, as determined by loss of fluorescence over a period of 30 min, was undetectable at both excitation wavelengths.

The ratio of the fluorescence intensity at the two excitation wavelengths can be converted into an estimate of ionized intracellular calcium concentration by use of the formula: $[Ca^{2+}]_i$= Kd x (Fmin/Fmax) x (R-Rmin)/(Rmax-R), where R, Rmin, and Rmax are the fluorescence ratios recorded during the experiment (R) and during calibration tests on unlysed cells using 4 mM ionomycin in SGNBS (Rmax), followed by 10 mM EGTA addition at pH 8.2 (Rmin). Fmin and Fmax are the corresponding fluorescence intensities for the 380 nm excitation and Kd is the Fura-2 dissociation constant at room temperature (135 nM).

Small volumes (10 - 50 µl) of drugs (hormones, agonists, neurotransmitters, TRH, bradykinin) from prediluted stock solutions prepared in the appropriate buffer solutions were directly added to the experimental chamber.

B. Results

Exposure of HEK293/TRHR-2 cells, but not wild type HEK 293 cells, to nanomolar concentrations of TRH resulted in a marked transient increase in the intracellular calcium concentration (see figure 5). The peak of $[Ca^{++}]_i$ concentration was reached after about 1 second and the baseline $[Ca^{++}]_i$ was attained after about 1 minute. TRH did not require the presence of extracellular calcium to evoke the transient raise in $[Ca^{++}]_i$.

Example 5: Northern Blot Analysis

A rat Multiple Tissue Northern blot (Clonetech (cat# 7764-1) was used to study the distribution of TRHR-2 in various tissues. The blot contained 2 mg of rat poly(A)$^+$ mRNA isolated from heart, brain, spleen, lung, liver, skeletal muscle, kidney and testes. The blot was first pre-hybridized at 42°C for three hours in a solution containing 50% formamide, 5x SSPE, 10x Denhardt's solution, 100 μg/ml sheared and denatured salmon sperm DNA and 2% SDS. A radiolabelled probe was prepared using Ready-to-go DNA labelling kit (Pharmacia Biotech Cat.#27-9251-01) and the full length cDNA of the TRHR-2. Hybridization was carried out at 42° C for about 18 hours in the solution described above. After an overnight hybridization, the blot was rinsed 2 times in 2x SSC, 0.05% SDS at room temperature and then washed 2 times for 15 minutes at room temperature followed by 2 washes at 50 °C for 15 minutes each and then 2 washes at 60 °C in the same solution. The blot was then exposed at -80 °C for 7 days to Kodak Biomax film with intensifying screens.

Expression of TRHR-2 mRNA was detected only in brain tissue (see fig 6). The apparent size of the mRNA is about 8 kb kilobases. Other tissues contained either no message or, at least, an insufficient amount of message to be detected after one week exposure under the conditions described.

Example 6: In Situ Hybridization

A. Hybridization Procedure

Animals and tissue preparation

Adult male Sprague-Dawley rats (~300 gm; Charles River, St-Constant, Quebec) were sacrificed by decapitation. Brain, pituitary and spinal cord were promptly removed, snap-frozen in isopentane at -40°C for 20 seconds and stored at -80°C. Frozen tissue was sectioned at 14 mm in a Microm HM 500 M cryostat (Germany) and thaw-mounted onto ProbeOn Plus slides (Fisher Scientific, Montreal, Quebec). Sections were stored at -80°C prior to in situ hybridization.

Riboprobe synthesis

The plasmid pCDNA3-TRHR2 was linearized using either XbaI or HindIII restriction enzymes, which cut in the polylinker on either side of the inserted cDNA. Sense and antisense TRHR-2 riboprobes were transcribed *in vitro* using either T7 or SP6 RNA polymerases (Pharmacia, Baie d'Urfe, Quebec), respectively in the presence of [^{35}S]UTP (~800 Ci/mmol; Amersham, Oakville, Ontario). Following transcription, the DNA template was digested with DNAse I (Pharmacia). Riboprobes were subsequently purified by phenol/chloroform/isoamyl alcohol extraction and precipitated in 70% ethanol containing ammonium acetate and tRNA. The quality of labeled riboprobes was verified by polyacrylamide-urea gel electrophoresis.

In situ Hybridization

Sections were postfixed in 4% paraformaldehyde (BDH, Poole, England) in 0.1 M phosphate buffer (pH 7.4) for 10 min at room temperature (RT) and rinsed in 3 changes of 2X standard sodium citrate buffer (SSC: 0.15 M NaCl. 0.015 M sodium citrate, pH 7.0).

Sections were then equilibrated in 0.1 M triethanolamine, treated with 0.25% acetic anhydride in triethanolamine, rinsed in 2X SSC and dehydrated in an ethanol series (50-100%). Hybridization was performed in a buffer containing 75% formamide (Sigma, St. Louis, Mo), 600 mM NaCl, 10 mM Tris (pH 7.5), 1 mM EDTA, 1X Denhardt's solution (Sigma), 50 μg/ml denatured salmon sperm DNA (Sigma), 50 μg/ml yeast tRNA (Sigma), 10% dextran sulfate (Sigma), 10 mM dithiothreitol and [^{35}S]UTP-labeled cRNA probes (10 X10^6 cpm/ml) at 55°C for 18 h in humidified chambers. Following hybridization, slides were rinsed in 2X SSC at RT, treated with 20 μg/ml RNase IA (Pharmacia) in RNase buffer (10 mM Tris, 500 mM NaCl, 1 mM EDTA, pH 7.5) for 45 min at RT and washed to a final stringency of 0.1X SSC at 65°C. Sections were then dehydrated and exposed to Biomax MR Kodak film for 10 days. Neuroanatomical structures were identified according to the Paxinos and Watson rat brain atlas (Paxinos *et al.*, The Rat Brain in Stereotaxic Coordinates, Academic Press (1986)).

B. Results

The most prominent labeling for TRHR-2 was detected throughout the thalamus, with the anterior, centromedian, centrolateral, paracentral and ventroposteromedian (VPM) nuclei exhibiting the highest intensity (see Table 2 and figure 6). In the more caudal thalamus, the medial geniculate nucleus was also moderately labeled. In addition, layers III - V of the cerebral cortex were moderately labeled. The pontine nuclei as well as the Purkinje cell layer of the cerebellum also displayed a high density of TRHR-2 mRNA hybridization. More moderate labeling was detected in the medial amygdalar nucleus as well as in a specific portion of the lateral hypothalamic area which does correspond to well known nuclear boundaries. Moderate to weak hybridization was detected throughout the reticular formation of the brain stem. Other cephalic areas such as the hippocampus, the remaining hypothalamus, the pituitary gland and basal ganglia were generally devoid of labeling. In the spinal cord, TRHR-2 mRNA expression was restricted to the entire dorsal horn. This is in stark contrast to that of the known TRH receptor mRNA under the same conditions

which appears to be present only in the hypothalamus, the anterior pituitary gland and some sparse neurons in the ventral horn of the spinal cord.

Table 2: Localization of TRHR-1 and TRHR-2 by In Situ Hybridization

Tissue		TRHR-1	TRHR-2
Pituitary Gland	anterior	+++	-
	intermediate	-	-
	posterior	-	-
Spinal cord	dorsal horn	-	++
	ventral horn	+	-
Brain	piriform cortex	+	-
	amygdala	+	+
	hypothalamic nuclei	+	-
	thalamus	-	+++
	medial habenular n.	-	++
	frontal & parietal cx.	-	++
	pontine nuclei	-	+++
	cerebellum	-	+++

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT: Astra Pharma Inc. Canada

(ii) TITLE OF INVENTION: New Receptor

(iii) NUMBER OF SEQUENCES: 4

(iv) CORRESPONDENCE ADDRESS:

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(C) CITY:

(D) STATE:

(E) COUNTRY: SWEDEN

(F) ZIP:

(v) COMPUTER READABLE FORM:

(A) MEDIUM TYPE: Floppy disk

(B) COMPUTER: IBM PC compatible

(C) OPERATING SYSTEM: PC-DOS/MS-DOS

(D) SOFTWARE: PatentIn Release #1.0, Version #1.30

(vi) CURRENT APPLICATION DATA:

(A) APPLICATION NUMBER:

(B) FILING DATE:

(C) CLASSIFICATION:

(viii) ATTORNEY/AGENT INFORMATION:

(A) NAME:

(B) REGISTRATION NUMBER:

(C) REFERENCE/DOCKET NUMBER:

(ix) TELECOMMUNICATION INFORMATION:

(A) TELEPHONE: 46-8 553 26000

(B) TELEFAX: 46-8 553 28820

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 1600 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: double

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

CTTTAAACCA CAGCCTCTCA AATACGCATC CCTACACTGG CTCCTTTCTT GGTCTTCCTA 60

TCTGAGCCCT GATGGCTTCT CCAGCTGCTC TTCCAGAGAC CTGGGTTCAA TTCCCAGCAC 120

CTATATGACA ACTTACAGAT TGGTGGCTGT AACTCCAATC CGGGGGATGC AATGCCATCT 180

TCTGGCCTCC AGAGGCACTA CATACACACA TGATACACAG AATATACACA CGTGTATATT 240

TAGGTAAAGT GCCTGTGCAC ATAAAAAAAA ATAAAAAGGA AAAAAATTAA ATCAGAAGGA 300

ACAGGCACCG GTCACTTACC AAGGTCAAGG CCTACAGGGC ACCACAGAAA ACACCAGCAA 360

GATGGATGGC CCCAGTAATG TCTCGCTCAT TCACGGTGAC ACCACGCTGG GCCTGCCAGA 420

GTACAAGGTG GTCTCAGTCT TCCTAGTGCT CCTGGTGTGC ACCCTGGGCA TCGTGGGCAA 480

TGCCATGGTG ATTCTGGTGG TGCTGACCTC ACGTGACATG CACACACCCA CCAACTGCTA 540

CCTGGTCAGC CTGGCCCTCG CTGACCTCCT CGTGCTGCTG GCTGCGGGTC TGCCCAATGT 600

CTCTGACAGC CTAGTGGGGC ACTGGATCTA TGGACGTGCT GGCTGCTTGG GCATCACCTA 660

CTTCCAGTAC CTGGGCATCA ATGTCTCCTC CTTCTCCATC CTGGCCTTCA CTGTGGAGAG 720

GTATATAGCC ATTTGCCACC CACTGAGAGC ACAGACCGTG TGCACTGTGG CCCGGGCCAA 780

ACGGATCATG GCAGGCATCT GGGGGGTCAC GTCCCTCTAT TGCCTACTCT GGTTCTTCCT 840

GGTGGATCTC AATGTCCGTG ACAACCAGCG CCTTGAATGT GGCTACAAAG TGCCCCGAGG 900

ACTCTACCTG CCCATCTACC TGCTGGACTT CGCTGTCTTT TTCATCGGAC CCTTGCTGGT 960

GACCCTCGTG CTCTATGGGC TCATCGGGAG GATTTTATTT CAGAGCCCGT TGTCCCAGGA 1020

AGCCTGGCAG AAGGAGAGGC AGCCCCATGG GCAGAGCGAG GCTGCACCAG GCAACTGCTC 1080

CAGGGCCAAG AGCTCCAGGA AGCAGGCCAC CAGGATGCTG GCCGTGGTTG TGTTGCTTTT 1140

TGCCGTGCTG TGGACCCCTT ACCGCACACT GGTACTGCTC AACTCCTTTG TGGCCCAGCC 1200

TTTCCTGGAC CCCTGGGTCC TGCTGTTCTG CCGCACCTGT GTCTACACCA ACAGCGCTGT 1260

CAACCCTGTC GTCTACAGCC TGATGTCACA GAAGTTCCGG GCGGCCTTCC TGAAACTGTG 1320

CTGGTGCAGG GCAGCTGGGC CACAGCGGAG GGCAGCACGC GTCCTCACCA GTAACTACAG 1380

TGCCGCCCAG GAGACCTCAG AAGGAACTGA GAAGATGTAG CTGGGCTCCA GTGAGGTCTC 1440

AGGTCCCACG GCAGCAGGTC CCCTGGCCTG TCAGCATGAG CCCTACTTCA GTGTGCTCTG 1500

AGGACTCCCG CCTGGCCCCT GACCCCGCTT AAGGCTTGGT TGGCATTTGG GAGGCATCAG 1560

GAGAGGGGCA GGCAGCTCCT TGCTTATGGG TTTCCAGAGG 1600

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 352 amino acids

(B) TYPE: amino acid

(C) STRANDEDNESS: not relevant

(D) TOPOLOGY: not relevant

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

Met Asp Gly Pro Ser Asn Val Ser Leu Ile His Gly Asp Thr Thr Leu
1 5 10 15

Gly Leu Pro Glu Tyr Lys Val Val Ser Val Phe Leu Val Leu Leu Val
20 25 30

Cys Thr Leu Gly Ile Val Gly Asn Ala Met Val Ile Leu Val Val Leu
35 40 45

Thr Ser Arg Asp Met His Thr Pro Thr Asn Cys Tyr Leu Val Ser Leu
50 55 60

Ala Leu Ala Asp Leu Leu Val Leu Leu Ala Ala Gly Leu Pro Asn Val
65 70 75 80

Ser Asp Ser Leu Val Gly His Trp Ile Tyr Gly Arg Ala Gly Cys Leu
85 90 95

Gly Ile Thr Tyr Phe Gln Tyr Leu Gly Ile Asn Val Ser Ser Phe Ser
100 105 110

Ile Leu Ala Phe Thr Val Glu Arg Tyr Ile Ala Ile Cys His Pro Leu
115 120 125

Arg Ala Gln Thr Val Cys Thr Val Ala Arg Ala Lys Arg Ile Met Ala
130 135 140

Gly Ile Trp Gly Val Thr Ser Leu Tyr Cys Leu Leu Trp Phe Phe Leu
145 150 155 160

Val Asp Leu Asn Val Arg Asp Asn Gln Arg Leu Glu Cys Gly Tyr Lys
165 170 175

Val Pro Arg Gly Leu Tyr Leu Pro Ile Tyr Leu Leu Asp Phe Ala Val
180 185 190

Phe Phe Ile Gly Pro Leu Leu Val Thr Leu Val Leu Tyr Gly Leu Ile
195 200 205

Gly Arg Ile Leu Phe Gln Ser Pro Leu Ser Gln Glu Ala Trp Gln Lys
210 215 220

Glu Arg Gln Pro His Gly Gln Ser Glu Ala Ala Pro Gly Asn Cys Ser
225 230 235 240

Arg Ala Lys Ser Ser Arg Lys Gln Ala Thr Arg Met Leu Ala Val Val
245 250 255

Val Leu Leu Phe Ala Val Leu Trp Thr Pro Tyr Arg Thr Leu Val Leu
260 265 270

Leu Asn Ser Phe Val Ala Gln Pro Phe Leu Asp Pro Trp Val Leu Leu
275 280 285

Phe Cys Arg Thr Cys Val Tyr Thr Asn Ser Ala Val Asn Pro Val Val
290 295 300

Tyr Ser Leu Met Ser Gln Lys Phe Arg Ala Ala Phe Leu Lys Leu Cys
305 310 315 320

Trp Cys Arg Ala Ala Gly Pro Gln Arg Arg Ala Ala Arg Val Leu Thr
325 330 335

Ser Asn Tyr Ser Ala Ala Gln Glu Thr Ser Glu Gly Thr Glu Lys Met
340 345 350

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 17 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

(A) DESCRIPTION: /desc = "SYNTHETIC PRIMER"

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(ix) FEATURE:

(A) NAME/KEY: modified_base

(B) LOCATION: group (9, 12)

(D) OTHER INFORMATION: /mod_base= i

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

ATYRSYRTRG ARMGRTA 17

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 20 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

(A) DESCRIPTION: /desc = "synthetic primer"

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(ix) FEATURE:

(A) NAME/KEY: modified_base

(B) LOCATION: group(11, 14)

(D) OTHER INFORMATION: /mod_base= i

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

MWGGYRTAGA RSARMGGRTT 20

What is Claimed is:

1. A protein, except as existing in nature, comprising the amino acid sequence consisting functionally of SEQ ID NO:2.

2. The substantially pure protein of claim 1, wherein said amino acid sequence consists essentially of the amino acid sequence of SEQ ID NO:2.

3. An antibody made by a process comprising the step of injecting a pharmaceutically acceptable preparation comprising the protein of either claim 1 or claim 2 into an animal capable of producing said antibody.

4. The process of claim 3, wherein said animal is a mouse and said process further comprises fusing spleen cells from said mouse with myeloma cells to produce a monoclonal antibody binding to said protein.

5. An antibody that binds preferentially to the protein of claim 2.

6. A substantially pure polynucleotide encoding a protein comprising the amino acid sequence consisting functionally of the sequence of SEQ ID NO:2.

7. The polynucleotide of claim 6 wherein said polynucleotide encodes a protein consisting essentially of the amino acid sequence of SEQ ID NO:2.

8. An expression vector comprising the polynucleotide of either claim 6 or claim 7.

9. A host cell transformed with the vector of claim 8.

10. Recombinant thyrotropin-releasing hormone receptor produced by the host cell of claim 9.

11. The polynucleotide of claim 7, wherein said polynucleotide has a sequence consisting essentially of that of SEQ ID NO:1.

12. An expression vector comprising the polynucleotide of claim 11.

13. A host cell transformed with the vector of claim 12.

14. A method for assaying a test compound for its ability to bind to the TRHR2 receptor, comprising:

a) incubating a source containing TRHR2 but substantially free of other receptors for TRH, with;

i) a ligand known to bind to TRHR2;

ii) said test compound; and

b) determining the extent to which said ligand binding is displaced by said test compound.

15. The method of claim 14, wherein said ligand is TRH.

16. The method of either claim 14 or claim 15, wherein said source of TRHR2 is a cell transformed with an expression vector comprising a polynucleotide encoding a protein consisting essentially of the amino acid sequence of SEQ ID NO:2.

17. The method of either claim 14 or claim 15, wherein said source of TRHR2 is a membrane preparation derived from a cell transformed with a vector for expressing TRHR2 and comprising a polynucleotide encoding a protein consisting essentially of the amino acid sequence of SEQ ID NO:2.

18. The method of claim 14, further comprising the step of measuring changes in second messenger concentration in response to ligand binding.

19. The method of claim 18, wherein said step comprises measuring changes in intracellular calcium concentration in response to ligand binding.

20. A method for assaying a test compound for its ability to alter the expression of TRHR2 comprising:

(a) growing cells expressing TRHR2 but substantially free of other TRH receptors in the presence of said test compound;

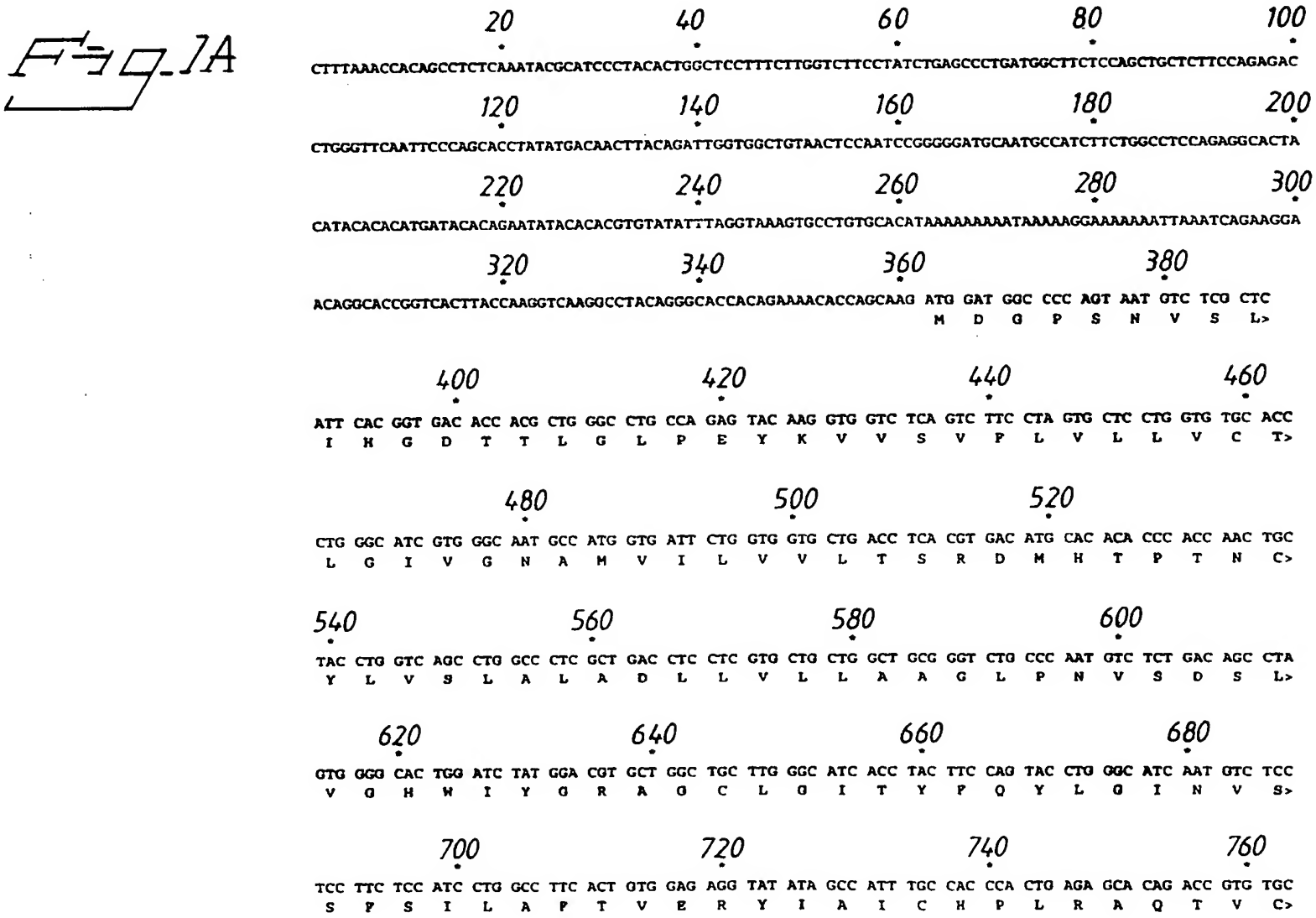
(b) collecting said cells; and

(c) comparing receptor expression in the cells exposed to said test compound with control cells grown under essentially identical conditions but not exposed to said test compound.

21. The method of claim 20, wherein said cells expressing TRHR2 are cells transformed with an expression vector comprising a polynucleotide sequence encoding a protein with an amino acid sequence consisting essentially of the sequence of SEQ ID NO:2.

22. The method of claim 21, wherein said receptor expression is determined by means of receptor binding assays

Fig. 1A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120 140 160 180 200
CTGGGTTCAATTCCCAGCACCTATATGACAACTTACAGATTGGTGGCTGTAACTCCAATCCGGGGGATGCAATGCCATCTTCTGGCCTCCAGAGGCACTA
220 240 260 280 300
CATACACACATGATACACAGAATATACACACGTGTATATTTAGGTAAAGTGCCTGTGCACATAAAAAAAAAATAAAAAGGAAAAAAATTAAATCAGAAGGA
320 340 360 380
ACAGGCACCGGTCACTTACCAAGGTCAAGGCCTACAGGGCACCACAGAAAACACCAGCAAG ATG GAT GGC CCC AGT AAT GTC TCG CTC
M D G P S N V S L>
400 420 440 460
ATT CAC GGT GAC ACC ACG CTG GGC CTG CCA GAG TAC AAG GTG GTC TCA GTC TTC CTA GTG CTC CTG GTG TGC ACC
I H G D T T L G L P E Y K V V S V F L V L L V C T>
480 500 520
CTG GGC ATC GTG GGC AAT GCC ATG GTG ATT CTG GTG GTG CTG ACC TCA CGT GAC ATG CAC ACA CCC ACC AAC TGC
L G I V G N A M V I L V V L T S R D M H T P T N C>
540 560 580 600
TAC CTG GTC AGC CTG GCC CTC GCT GAC CTC CTC GTG CTG CTG GCT GCG GGT CTG CCC AAT GTC TCT GAC AGC CTA
Y L V S L A L A D L L V L L A A G L P N V S D S L>
620 640 660 680
GTG GGG CAC TGG ATC TAT GGA CGT GCT GGC TGC TTG GGC ATC ACC TAC TTC CAG TAC CTG GGC ATC AAT GTC TCC
V G H W I Y G R A G C L G I T Y F Q Y L G I N V S>
700 720 740 760
TCC TTC TCC ATC CTG GCC TTC ACT GTG GAG AGG TAT ATA GCC ATT TGC CAC CCA CTG AGA GCA CAG ACC GTG TGC
S F S I L A F T V E R Y I A I C H P L R A Q T V C>
SUBSTITUTE SHEET (RULE 26)

Fig.1A

```
                                780                             800                             820
ACT GTG GCC CGG GCC AAA CGG ATC ATG GCA GGC ATC TGG GGG GTC ACG TCC CTC TAT TGC CTA CTC TGG TTC TTC
 T   V   A   R   A   K   R   I   M   A   G   I   W   G   V   T   S   L   Y   C   L   L   W   F   F>

840                             860                             880                             900
CTG GTG GAT CTC AAT GTC CGT GAC AAC CAG CGC CTT GAA TGT GGC TAC AAA GTG CCC CGA GGA CTC TAC CTG CCC
 L   V   D   L   N   V   R   D   N   Q   R   L   E   C   G   Y   K   V   P   R   G   L   Y   L   P>

                920                             940                             960                             980
ATC TAC CTG CTG GAC TTC GCT GTC TTT TTC ATC GGA CCC TTG CTG GTG ACC CTC GTG CTC TAT GGG CTC ATC GGG
 I   Y   L   L   D   F   A   V   F   F   I   G   P   L   L   V   T   L   V   L   Y   G   L   I   G>

                1000                            1020                            1040                            1060
AGG ATT TTA TTT CAG AGC CCG TTG TCC CAG GAA GCC TGG CAG AAG GAG AGG CAG CCC CAT GGG CAG AGC GAG GCT
 R   I   L   F   Q   S   P   L   S   Q   E   A   W   Q   K   E   R   Q   P   H   G   Q   S   E   A>

                                1080                            1100                            1120
GCA CCA GGC AAC TGC TCC AGG GCC AAG AGC TCC AGG AAG CAG GCC ACC AGG ATG CTG GCC GTG GTT GTG TTG CTT
 A   P   G   N   C   S   R   A   K   S   S   R   K   Q   A   T   R   M   L   A   V   V   V   L   L>

1140                            1160                            1180                            1200
TTT GCC GTG CTG TGG ACC CCT TAC CGC ACA CTG GTA CTG CTC AAC TCC TTT GTG GCC CAG CCT TTC CTG GAC CCC
 F   A   V   L   W   T   P   Y   R   T   L   V   L   L   N   S   F   V   A   Q   P   F   L   D   P>

                1220                            1240                            1260                            1280
TGG GTC CTG CTG TTC TGC CGC ACC TGT GTC TAC ACC AAC AGC GCT GTC AAC CCT GTC GTC TAC AGC CTG ATG TCA
 W   V   L   L   F   C   R   T   C   V   Y   T   N   S   A   V   N   P   V   V   Y   S   L   M   S>

                1300                            1320                            1340                            1360
CAG AAG TTC CGG GCG GCC TTC CTG AAA CTG TGC TGG TGC AGG GCA GCT GGG CCA CAG CGG AGG GCA GCA CGC GTC
 Q   K   F   R   A   A   F   L   K   L   C   W   C   R   A   A   G   P   Q   R   R   A   A   R   V>
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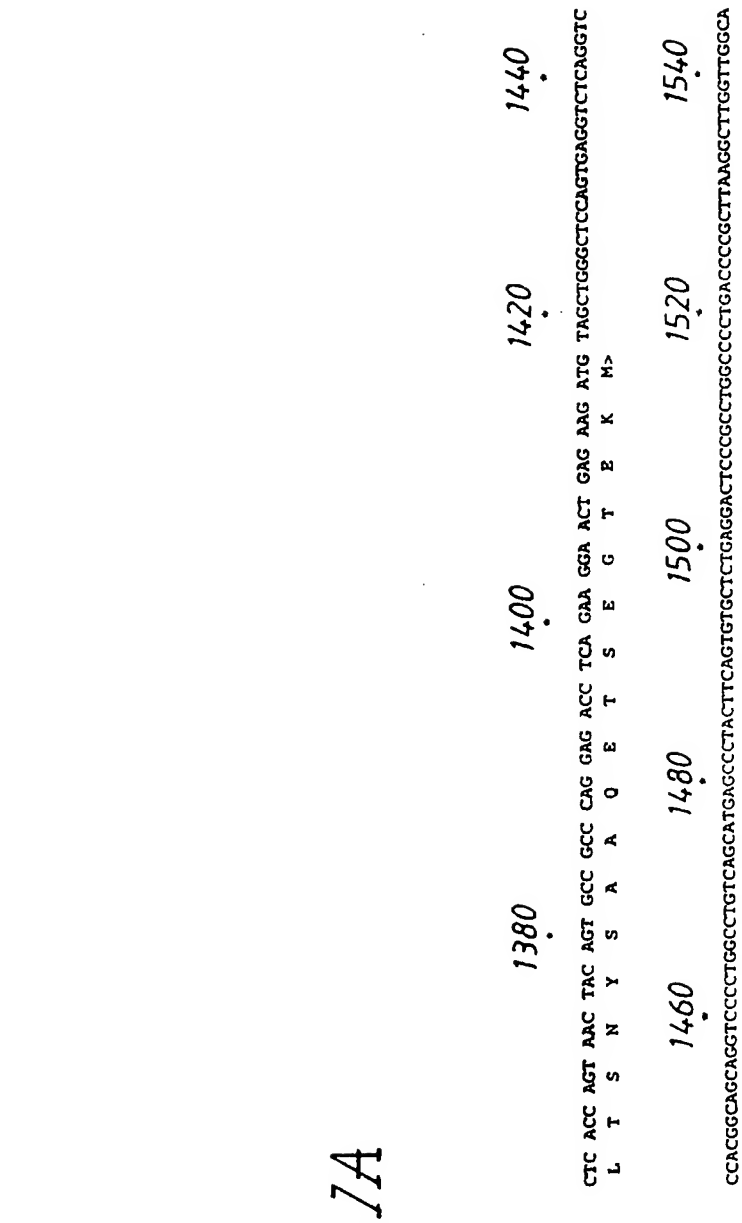
1380
1400
1420
1440
CTC ACC AGT AAC TAC AGT GCC GCC CAG GAG ACC TCA GAA GGA ACT GAG AAG ATG TAGCTGGGCTCCAGTGAGGTCTCAGGTC
L T S N Y S A A Q E T S E G T E K M>
1460
1480
1500
1520
1540
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1560
1580
1600
TTTGGGAGGCATCAGGAGAGGGGCAGGCAGCTCCTTGCTTATGGGTTTCCAGAGG

FIG. 1A

Fig. 1B

TRHR2 Amino Acid Sequence Range: 1 to 352

```
        10        20        30        40        50        60        70
MDGPSNVSLI HGDTTLGLPE YKVVSVFLVL LVCTLGIVGN AMVILVVLTS RDMHTPTNCY LVSLALADLL
        80        90       100       110       120       130       140
VLLAAGLPNV SDSLVGHWIY GRAGCLGITY FQYLGINVSS FSILAFTVER YIAICHPLRA QTVCTVARAK
       150       160       170       180       190       200       210
RIMAGIWGVT SLYCLLWFFL VDLNVRDNQR LECGYKVPRG LYLPIYLLDF AVFFIGPLLV TLVLYGLIGR
       220       230       240       250       260       270       280
ILFQSPLSQE AWQKERQPHG QSEAAPGNCS RAKSSRKQAT RMLAVVVLLF AVLWTPYRTL VLLNSFVAQP
       290       300       310       320       330       340       350
FLDPWVLLFC RTCVYTNSAV NPVVYSLMSQ KFRAAFLKLC WCRAAGPQRR AARVLTSNYS AAQETSEGTE
KM
```

Fig. 2

Alignement of the amino acid sequence of TRHR 2 with previously known TRHR1 receptor sequences

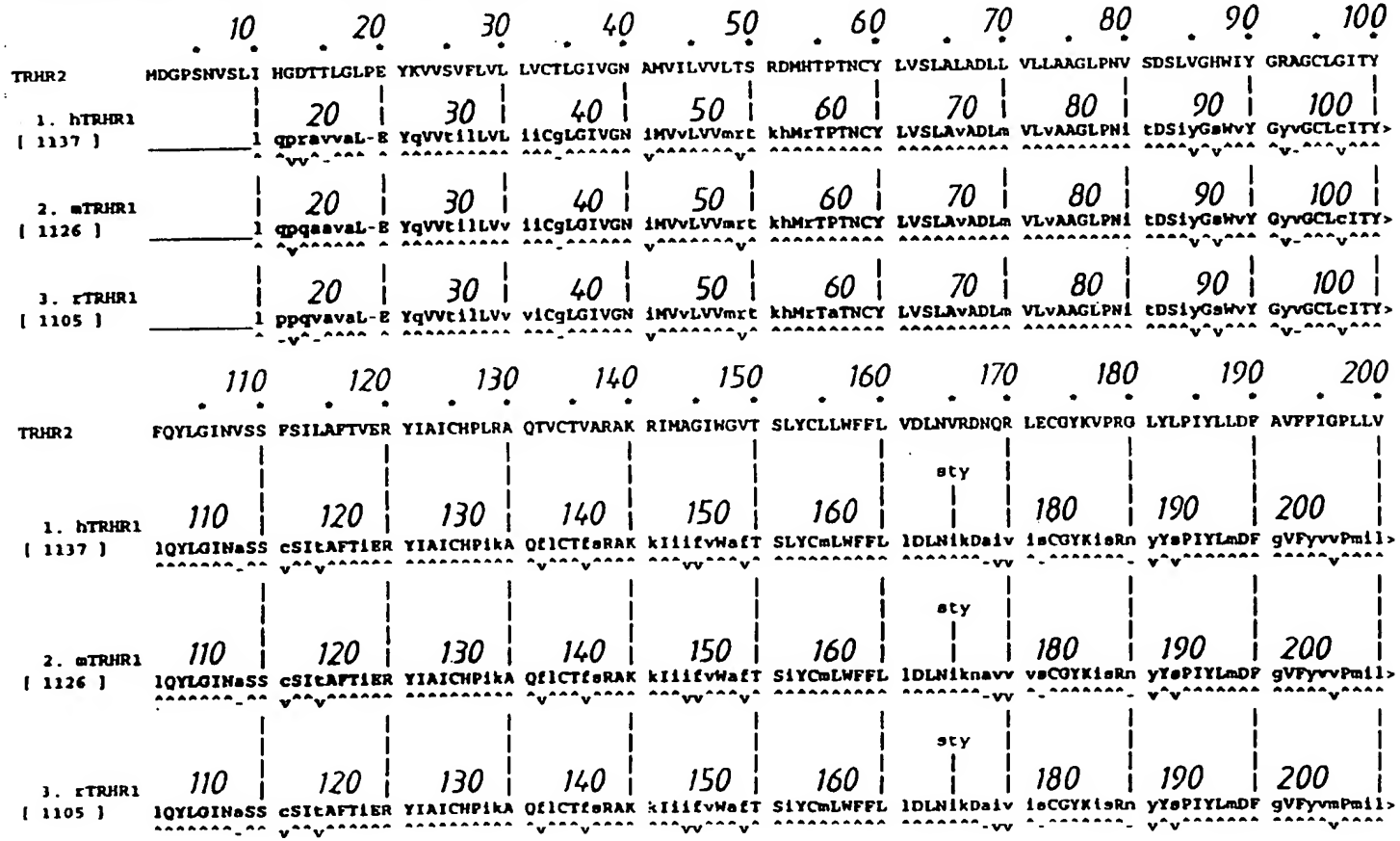


FIG. 2

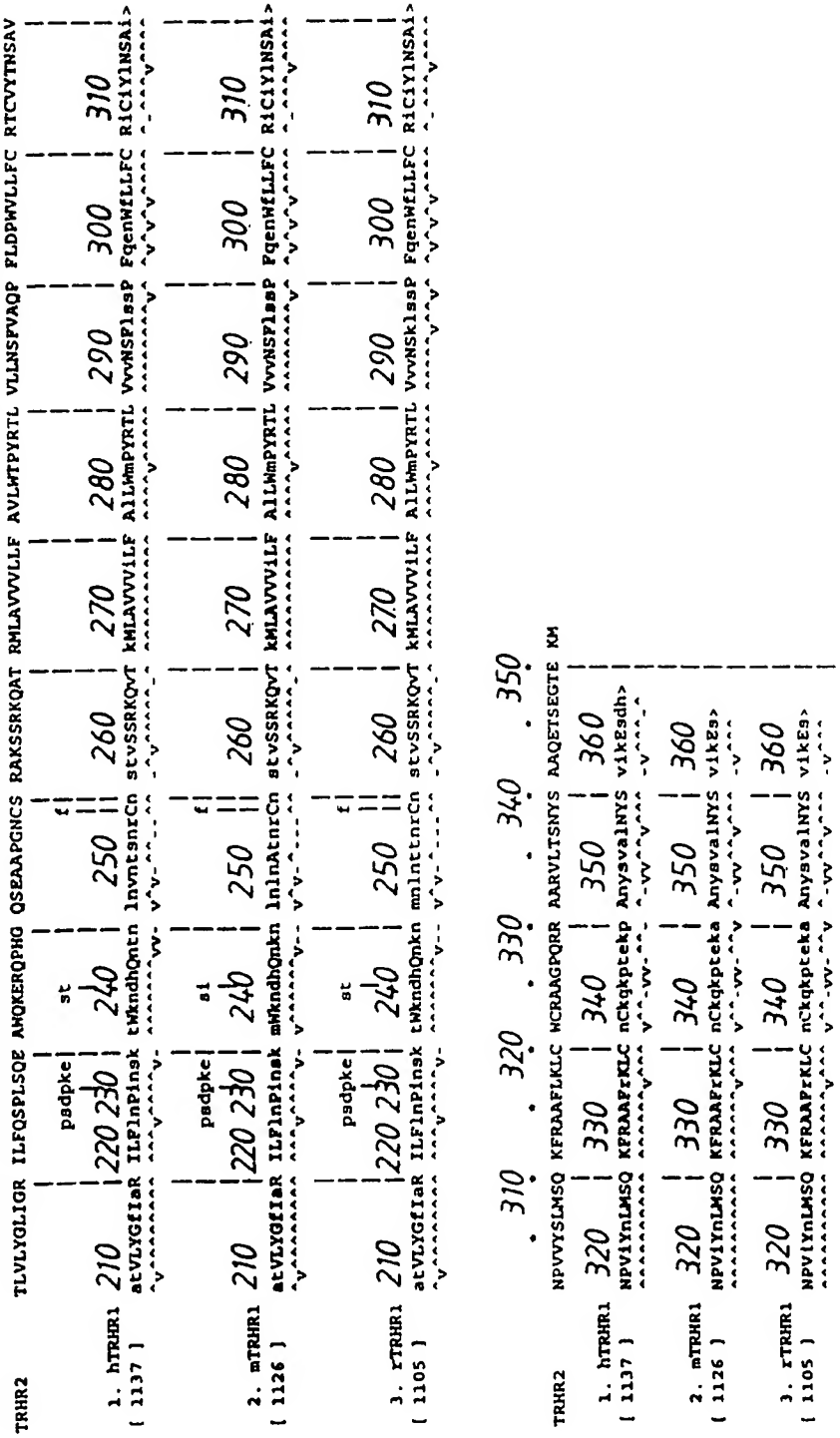


Fig.3

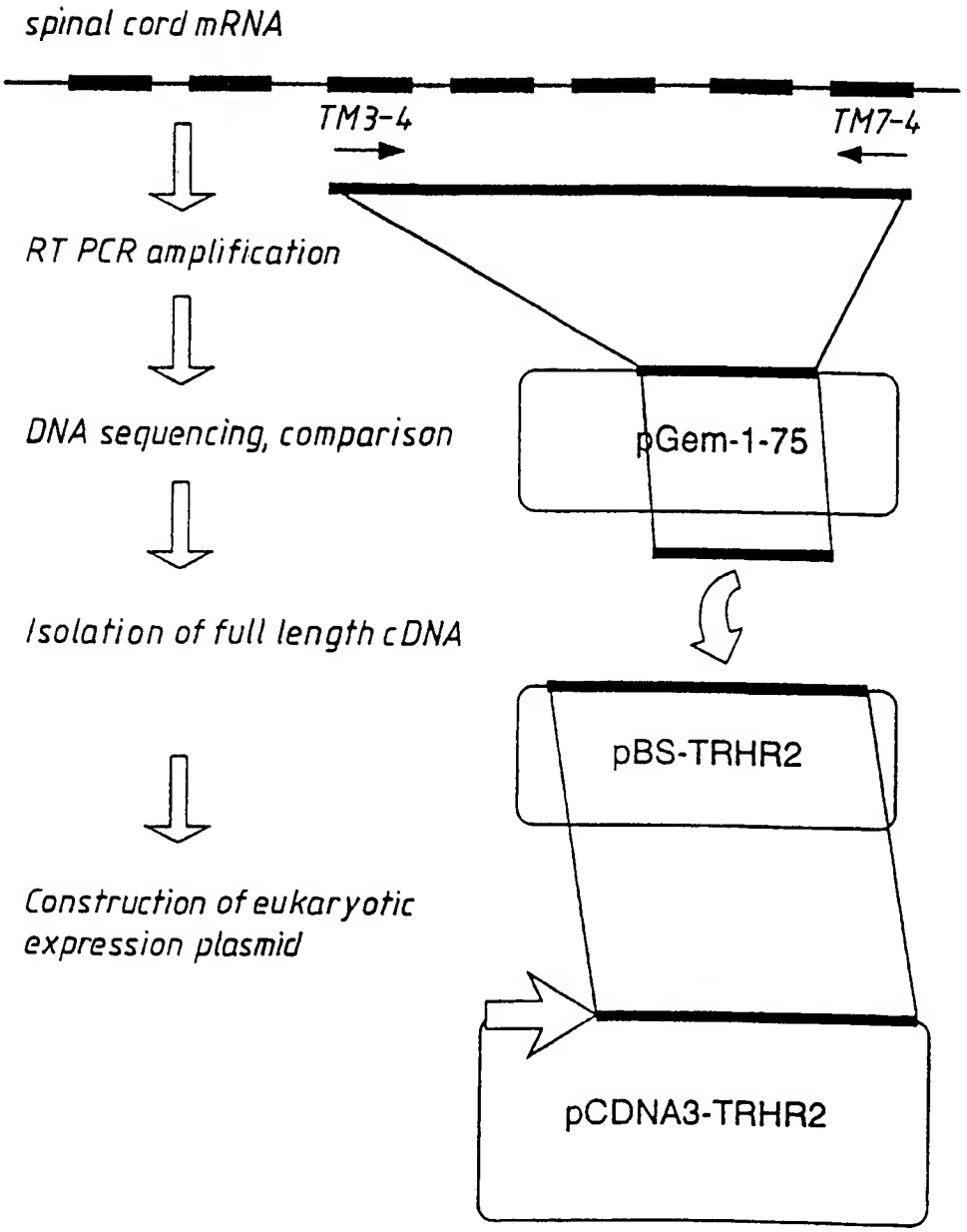
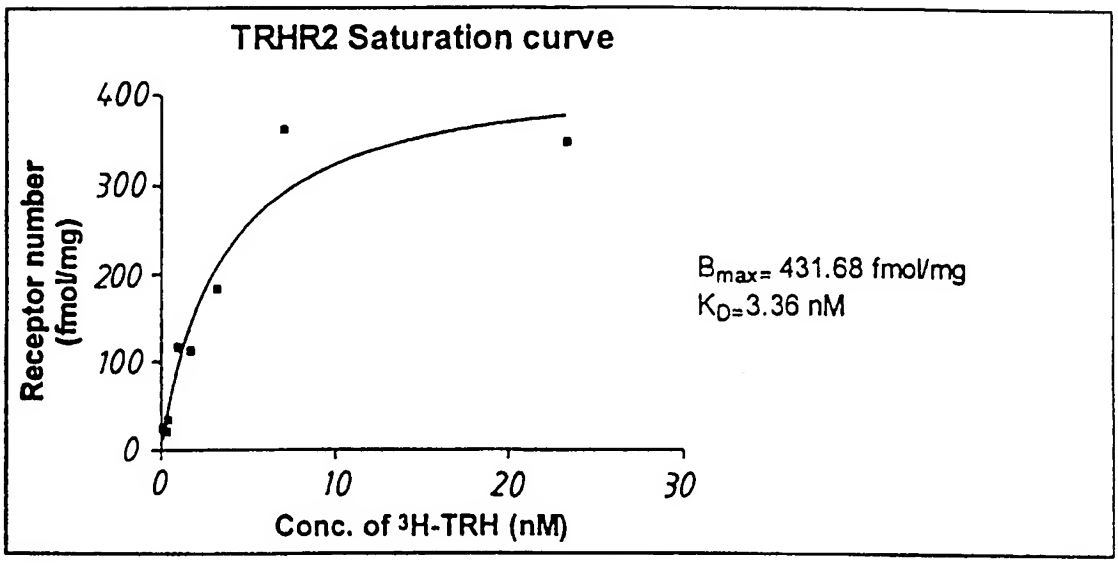

spinal cord mRNA
TM3-4
TM7-4
RT PCR amplification
DNA sequencing, comparison
pGem-1-75
Isolation of full length cDNA
pBS-TRHR2
Construction of eukaryotic expression plasmid
pCDNA3-TRHR2

Fig. 4

A


TRHR2 Saturation curve
Receptor number (fmol/mg)
400
300
200
100
0
0
10
20
30
Conc. of 3H-TRH (nM)
Bmax= 431.68 fmol/mg
KD=3.36 nM

B

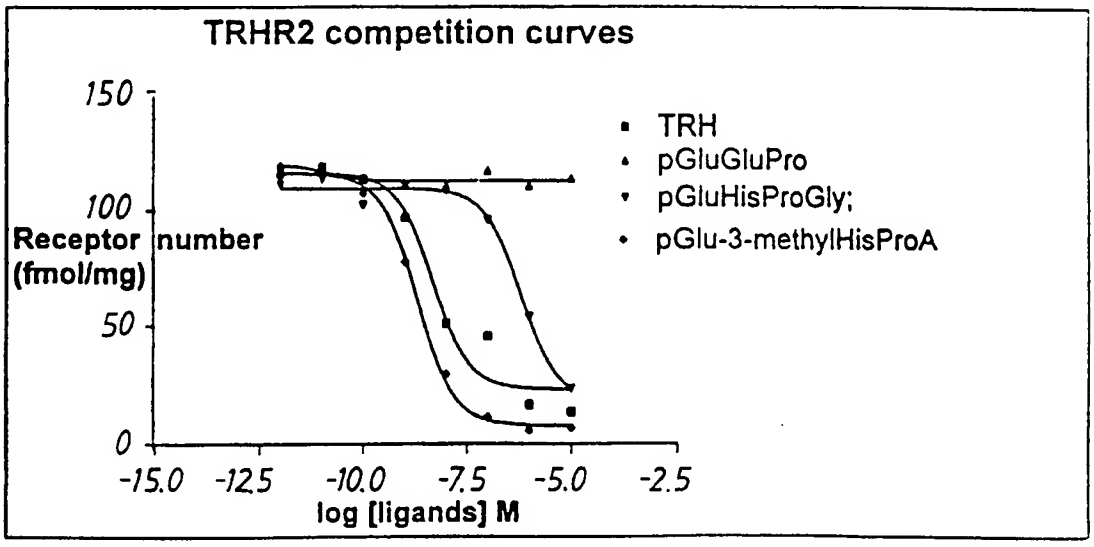

TRHR2 competition curves
150
100
50
0
Receptor number (fmol/mg)
-15.0
-12.5
-10.0
-7.5
-5.0
-2.5
log [ligands] M
TRH
pGluGluPro
pGluHisProGly;
pGlu-3-methylHisProA

Fig.5

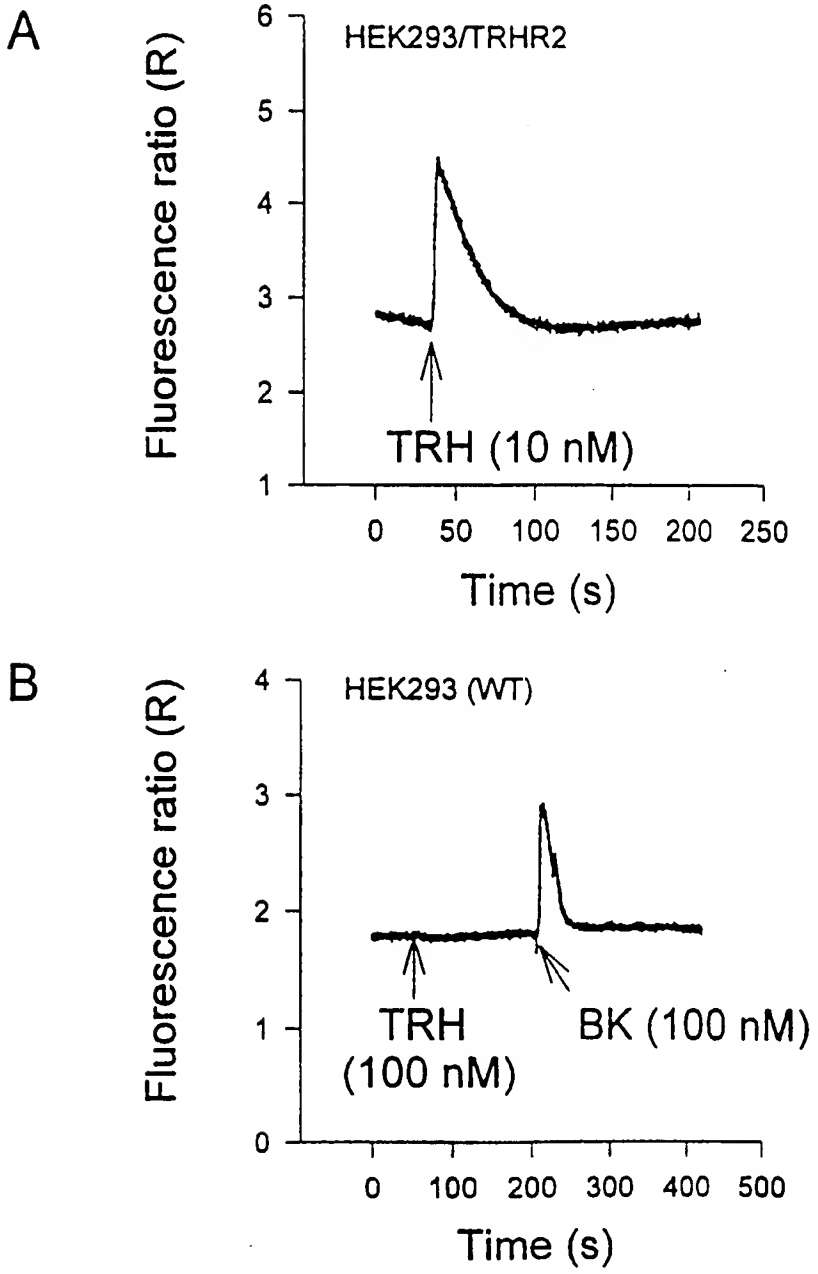

A
HEK293/TRHR2
Fluorescence ratio (R)
6
5
4
3
2
1
TRH (10 nM)
0
50
100
150
200
250
Time (s)
B
HEK293 (WT)
Fluorescence ratio (R)
4
3
2
1
0
TRH
(100 nM)
BK (100 nM)
0
100
200
300
400
500
Time (s)

Fig. 6

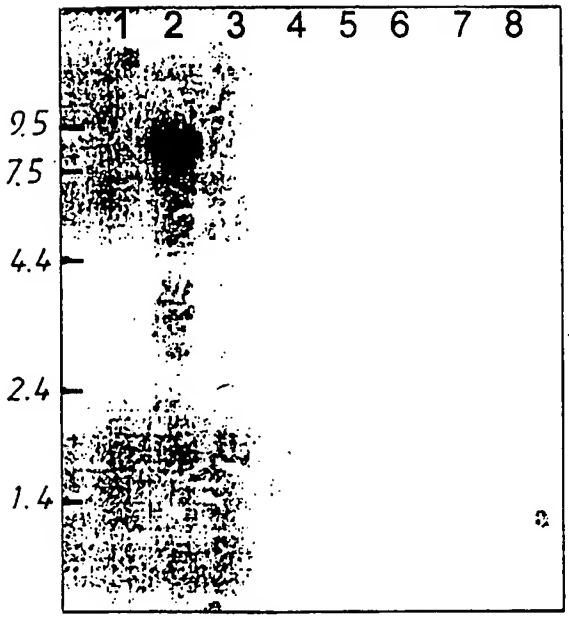
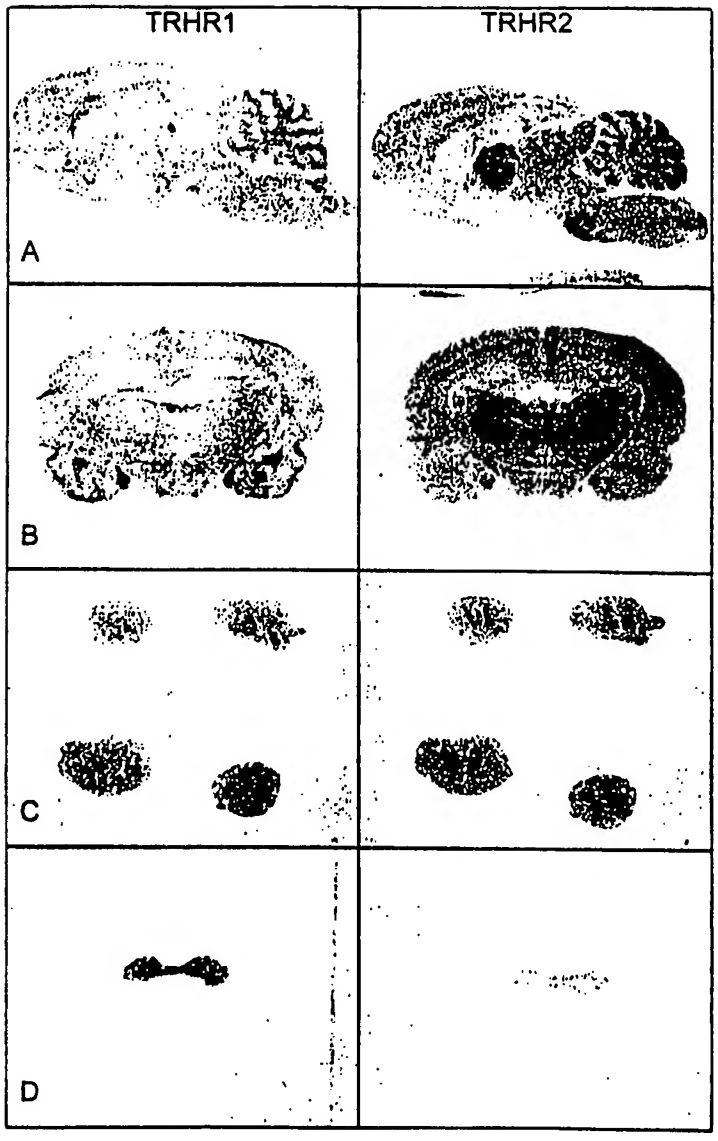


Fig. 7



INTERNATIONAL SEARCH REPORT

International application No.
PCT/SE 97/01999

A. CLASSIFICATION OF SUBJECT MATTER

IPC6: C07K 14/72, C07K 16/28
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC6: C12N, C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

SE,DK,FI,NO classes as above

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

WPI, MEDLINE, SCISEARCH, BIOSIS, DBA, CA

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 9429447 A2 (MEDICAL RESEARCH COUNCIL), 22 December 1994 (22.12.94) --	1-22
X	WO 9210565 A (CORNELL RESEARCH FOUNDATION, INC.), 25 June 1992 (25.06.92) --	1-22
A	Dialog Information Services, File 154, Medline, Dialog accession no. 07818456, Medline accession no. 93226257, Kaji H et al: "The regional distribution of thyrotropin-relasing hormone receptor messenger ribonucleic acid in the brain"; & Neurosci Lett (NETHERLANDS) Mar 5 1993, 151 (1) p 81-4 -- --------	1-22

☐ Further documents are listed in the continuation of Box C. ☒ See patent family annex.

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" erlier document but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance: the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance: the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
9 March 1998	11 -03- 1998
Name and mailing address of the ISA/ Swedish Patent Office Box 5055, S-102 42 STOCKHOLM Facsimile No. +46 8 666 02 86	Authorized officer Patrick Andersson Telephone No. +46 8 782 25 00

INTERNATIONAL SEARCH REPORT

Information on patent family members

03/02/98

International application No.
PCT/SE 97/01999

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9429447 A2	22/12/94	AU 7002594 A	03/01/95
		EP 0701610 A	20/03/96
		JP 9501310 T	10/02/97
WO 9210565 A	25/06/92	US 5288621 A	22/02/94